

## Alterations in plasma nitric oxide during aging in humans

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Nitric oxide (NO) is relatively harmless, but along with superoxide radical becomes precursor of many toxic species, such as peroxy and hydroxyl radicals, hydrogen peroxide, and peroxynitrite. In the present study, we determined plasma NO as a function of human age and correlated NO levels with total antioxidant capacity of the plasma. Results showed significant increase in NO level as a function of human age and plasma NO level positively correlated with total antioxidant potential. Increased NO may contribute to the development of oxidative stress during aging.

**Keywords:** Nitric oxide, Aging, Human, Oxidative stress

Aging is a multi-factorial process, involving morphological and biochemical changes in single cell and in the whole organism. Although the exact mechanism underlying aging is not well understood, evidences suggest a possible relationship between life span and production of free radicals<sup>1</sup>. The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has long been proposed as leading to random deleterious modifications of macromolecules with an associated progressive development of age-associated systemic disease<sup>2</sup>. Aerobic cells produce ROS as a byproduct of their metabolic processes. ROS cause oxidative damage to macromolecules, under conditions when the antioxidant defence of the body is overwhelmed<sup>3</sup>. A certain amount of oxidative damage takes place even under normal conditions, however, the rate of this damage increases during the aging process, as the efficiency of antioxidative and repair mechanisms decrease<sup>4,5</sup>.

Although nitric oxide is highly reactive having half-life of few seconds, yet it can diffuse cell membrane freely which makes it ideal for a transient

signal molecule. It is known to be involved in various age-associated diseases like atherosclerosis, hypertension etc<sup>6,7</sup> and in many other biological effects such as blood vessel dilatation, signalling<sup>8</sup>, neurotransmission<sup>9</sup>, regulation of hair follicle activity<sup>10</sup>, immune response<sup>11</sup>, and penile erection<sup>12</sup>. NO contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium<sup>13</sup>, however, the role of NO in human aging is not well understood yet.

Earlier, we reported age-dependent alterations in antioxidant enzymes<sup>14</sup> and some biomarkers of oxidative stress<sup>15</sup>, and a significant age-dependent decline in plasma antioxidant capacity, measured in terms of ferric reducing ability of the plasma (FRAP) values<sup>16</sup>. Since antioxidant capacity of the plasma is related to dietary intake of antioxidants, it is important to study the correlation between antioxidant capacity of the plasma and markers of oxidative stress. In the present study, we report the age-dependent alteration in the NO level. We have correlated it with total plasma antioxidant potential, measured in terms of FRAP values.

## Materials and Methods

### Subjects

The study was carried out on 42 normal healthy subjects of both sexes between the ages of 18-82 yrs. The subjects were screened for diabetes mellitus, asthma, tuberculosis or any other major illness. None of the subjects were smokers or taking any medication. All subjects gave their informed consent for the use of their blood samples for the study. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee. Human venous blood from different healthy volunteers was obtained by venipuncture. The blood was centrifuged at  $1800 \times g$  for 10 min at 4°C and plasma was collected.

### Determination of NO

NO was measured as nitrite by Griess reagent as described<sup>17</sup>. The Griess reagent contained 0.1% naphthalene diamine dihydrochloride and 1% sulphanilamide in 2.5% meta-phosphoric acid. These two solutions were mixed in 1:1 ratio at the time of

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assay. Sodium nitrite was used as standard and the sample readings were calculated by plotting standard curve. The values were expressed in  $\mu\text{M}$  nitrite produced. Absorbance was measured at 540 nm.

#### Determination of FRAP

The ferric reducing ability of plasma (FRAP) values were determined as described previously<sup>18</sup>. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-tri[2-pyridyl]-s-triazine (10 mM in 40 mM HCl) solution and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (20 mmol/L) solution in 10:1:1 ratio respectively. FRAP reagent (3 ml) was mixed with 100  $\mu\text{l}$  of plasma and the content was mixed vigorously. The absorbance was read at 593 nm at the interval of 30 s for 4 min. Aqueous solutions of known  $\text{Fe}^{2+}$  concentration in the range of 100-1000  $\mu\text{mol/L}$  were used for calibration. Using the regression equation, the FRAP values ( $\mu\text{mol Fe(II)}$  per L) of the plasma were calculated.

Statistical analyses were performed using the software PRISM 4. Relationships between various parameters were assessed using Pearson correlation coefficient ( $r$ ).

#### Results and Discussion

Oxidative stress has been reported to increase with aging<sup>15,16</sup>. We show here an age-dependent decrease in plasma antioxidant capacity, measured in terms of FRAP values. Similar findings have been reported earlier in relation to plasma membrane redox system (PMRS) and markers of oxidative stress in erythrocytes during human aging<sup>15,16</sup>. Although the importance of NO in aging has been reported in different mammals<sup>19-21</sup>, the data in healthy humans remain controversial and age-dependent studies are few<sup>11,22</sup>.

Fig. 1(a) shows the age-dependent increase in the level of plasma NO in humans. To analyze the correlation of plasma NO with plasma antioxidant capacity, we plot a quotient: NO/FRAP as a function of human age. Fig. 1b shows a strong positive correlation ( $r = 0.889$ ) between NO and total plasma antioxidant potential. Our results thus confirm that human aging is associated with increase in the NO level, which correlates with decrease in antioxidant potential. The increased plasma NO level, as a function of human age may be one possible cause for an increased oxidative stress during old age.

Nitric oxide combines with the superoxide ion to produce peroxynitrite which can react with intracellular targets, causing oxidation, nitration, or

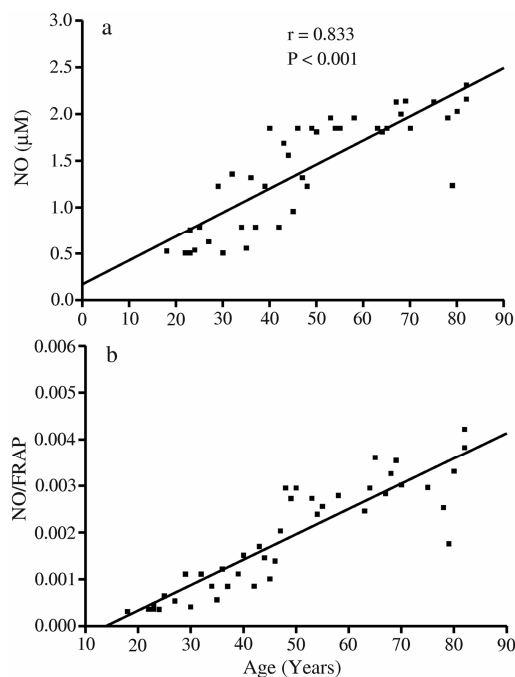


Fig. 1(a)—Plasma NO plotted as a function of age. Concentration of NO is expressed as  $\mu\text{M}$  nitrite produced; and (b) Plot of quotient (NO/FRAP values) as a function of age. FRAP values expressed as  $\mu\text{mol Fe(II)}$  per liter of plasma from Rizvi *et al*<sup>15</sup>

triggering cellular signals. The formation of peroxynitrite depends on concentration of both NO and superoxide anion and therefore, on the activities of both nitric oxide synthase (NOS) and superoxide dismutase. Aging is associated with increased production of free radicals, thus increased plasma NO may lead to formation of peroxynitrite. Another important physiological function of superoxide and NO is their competition for the interaction with mitochondrial cytochrome *c* oxidase. Disturbance of superoxide/NO balance leads to the dysfunction of mitochondria and the enhancement of apoptosis and oxidative stress, which are major causes of various pathological disorders and aging<sup>7</sup>.

In conclusion, the results of present study demonstrate significant age-related changes in plasma NO level and a positive correlation of these changes with the age-dependent decline in the total antioxidant capacity of the plasma. Increased NO may contribute to the development of oxidative stress during aging. These findings emphasize the need to establish age-dependent reference values for oxidative stress markers.

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